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Paraoxonase-2: A potential biomarker for skin cancer aggressiveness

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# TITLE PAGE

# Title

Paraoxonase-2: a potential biomarker for skin cancer aggressiveness.

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## ABSTRACT

**Background.** Cutaneous neoplasms include melanoma and non-melanoma skin cancers (NMSCs). Among NMSCs, basal cell carcinoma (BCC) represents the most common lesion. On the contrary, although accounting for less than 5% of all skin cancers, melanoma is responsible for most of cutaneous malignancy related-deaths.

Paraoxonase-2 (PON2) is an intracellular enzyme exerting a protective role against production of reactive oxygen species within mitochondrial respiratory chain. Recently, a growing attention has been focused on exploring the role of PON2 in cancer. The aim of this study was to investigate the diagnostic and prognostic role of PON2 in skin neoplasms.

**Matherials and methods.** 36 cases of BCC, distinguished between nodular and infiltrative lesions, as well as 29 melanoma samples were analyzed by immunohistochemistry to evaluate PON2 protein expression. Subsequent statistical analyses were carried out to explore the existance of correlations between intratumor enzyme levels and clinicopathological features.

**Results.** Results obtained showed PON2 overexpression in BCCs compared with controls. In particular, distinguishing between less and more aggressive tumor forms, we found no significant differences in enzyme levels between nodular BCCs and controls. Conversely, PON2 expression was significantly higher in infiltrative BCCs compared with controls. Moreover, the enzyme was strongly upregulated in melanoma samples with respect to controls. Interestingly, PON2 levels were positively correlated with Breslow thickness, Clark level, regression, mitoses, lymph node metastases, primary tumor (pT) parameter and pathological stage.

**Conclusions.** Reported findings seem to suggest that PON2 expression levels could be positively related with tumour aggressiveness of both BCC and melanoma.

### **KEYWORDS**

Paraoxonase-2, skin cancers, basal cell carcinoma, melanoma, immunohistochemistry, tumor biomarker.

## INTRODUCTION

Skin cancers are the most common malignacies in the white population worldwide<sup>1</sup> and represent a heterogeneous group of neoplasms, including cutaneous melanoma and non-melanoma skin cancers (NMSC).<sup>2</sup> NMSCs refer to carcinomas arising from keratinocyte malignant transformation<sup>2</sup> and comprise basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which accounts for 70% and 25% of all NMSCs, respectively.<sup>3</sup> The main risk factor involved in their pathogenesis is exposure to ultraviolet radiation.<sup>4</sup> Both BCC and SCC display a favourable prognosis if detected at early stage.<sup>3</sup>

BCC derives from basaloid cells and displays several histological variants, each characterized by different behavior and prognosis.<sup>2</sup> Nodular BCC is the most common type, accounting for 50% of all BCCs. and is characterized by the presence of large aggregates of cancer cells displaying well-defined borders.<sup>2,5</sup> This BCC subtype belongs to the low-risk group, due to its poor tendency to recurr or metastazise upon surgical excision.<sup>5</sup> On the contrary, infiltrative BCC represents an aggressive neoplastic variant, in which tumor causes invasion and destruction of surrounding tissues, such as sub-cutis and muscle.<sup>5,6</sup> However, even though tissue involvement might be extensive, metastastatic spread is a rare event.<sup>5</sup>

Melanoma develops from cancerous growth of melanocytes and represents the most aggressive and deadly lesion, among skin cancers.<sup>7</sup> Its lethality is due to the fact that, although comprising less than 5% of all skin neoplasms, it accounts for more than 75% of all skin-cancer-related deaths.<sup>4,7</sup> Early detection is therefore crucial for melanoma prognosis, since the estimated 5-year survival drops from over 99%, for those lesions diagnosed at early stage, to about 14%, for advanced stage diseases.<sup>8</sup>

Paraoxonase-2 (PON2) belongs to the multigene family of paraoxonases (PONs), also including paraoxonase-1 (PON1) and paraoxonase-3 (PON3). While PON1 and PON3 are mainly expressed in the liver, secreted into the plasma and associated with lipoproteins, PON2 displays an ubiquitous

expression pattern and remains inside the cell upon translation.<sup>9</sup> PON2 was found to be constitutively expressed in vascular cells, where it exerts antioxidant properties.<sup>10</sup> Within the cell, the enzyme is located in the nuclear envelope, endoplasmic reticulum (ER),<sup>11</sup> mitochondria<sup>12,13</sup> and plasma membrane.<sup>14</sup> PON2 anti-oxidative effect is due to its ability to reduce reactive oxygen species (ROS) production, thus counteracting intracellular oxidative stress.<sup>13</sup> Recent studies have been carried out to speculate the role of PON2 in cancer, thus disclosing enzyme overexpression in some solid tumors, such as oral,<sup>15</sup> bladder,<sup>16</sup> pancreatic,<sup>17</sup> ovarian<sup>18</sup> and gastric<sup>19</sup> cancer.

To date, there are no data dealing with PON2 expression in skin cancers, as well as the role played by the enzyme in these neoplasms. Therefore, the aim of the present study was to evaluate PON2 immunohistochemical expression in BCC and melanoma, as well as to explore the existence of correlations between enzyme levels and main prognostic parameters.

#### **MATERIALS AND METHODS**

#### Patients and tissue specimens

92 formalin-fixed and paraffin-embedded (FFPE) tissue specimens, collected between February 2018 and February 2020, were obtained from the archives of Pathology (Department of Biomedical Sciences and Public Health, Polytechnic University of Marche). Patients with familial or multiple melanoma cases, patients with nevoid basal cell carcinoma syndrome, and cases presenting other cancers were excluded. This retrospective study was carried out in compliance with the Declaration of Helsinki.

BCC group (including tumors and healthy tissue margins) consisted of 36 cases (23 males and 13 females; age range: 41-83; mean age: 68) and included 17 nodular and 19 infiltrative subtypes (Table 1, left column). Melanoma group was composed of 29 primary lesions (18 males and 11 females; age range: 28-96; mean age: 61) (Table 1, central column), while controls were 27 age-and gender-matched benign compound or dermal melanocytic nevi (Table 1, right column). Reporting of the study conforms to broad EQUATOR guidelines, as reported by Simera et al.<sup>20</sup>

#### Immunohistochemistry

PON2 expression in tumor and control tissues was assessed by immunohistochemical analyses.<sup>21</sup> 5μm sections were cut from FFPE blocks, mounted on poly-L-lysine-coated glass slides, deparaffinized in xylene, rehydrated in a graded alcohol series and treated with EnVision FLEX Target Retrieval Solution High pH (cat. GV804, lot. 20080562, Dako, Carpinteria, California, USA). Samples were then incubated with a 3% H<sub>2</sub>O<sub>2</sub> solution for 7 minutes to inhibit endogenous peroxidase and blocked with 5% Normal Goat Serum (cat. X0907, lot. 20083086, Dako). After washing with EnVision FLEX Wash Buffer (cat. GC807, lot. 20077433, Dako) for 5 minutes, sections were incubated with rabbit polyclonal antibody against human PON2 (1:1000 dilution) (cat. SAB1303623, lot. SA100914AJ, Sigma-Aldrich, St. Louis, Missouri, USA) at room temperature for 1h in a humified atmosphere. Samples were then washed, treated with EnVision

FLEX/HRP (cat. DM842, lot. 20078532, Dako) for 20 minutes and incubated with FLEX DAB+ Chromogen (cat. DM847, lot. 20080353, Dako) for 10 minutes, after a further washing. Following the counterstaining with Mayer's hematoxylin, sections were permanently mounted on glass slides. Human kidney tissue was used as a positive control, whereas negative control slides were obtained by replacing primary antibody with Rabbit IgG Isotype (cat. 10500C, lot. AB\_2532981, ThermoFisher Scientific, Waltham, Massachusetts, USA). Images depicting negative e positive

controls were reported in Supplementary Figure 1.

Specimens were simultaneously evaluated by two investigators blinded to the patient group, by using a double-headed light microscope equipped with a Nikon DS-Vi1 digital camera (Nikon Instruments, Europe BV, Kingston, Surrey, England). Agreement between observers was always >95%. Cell counting was performed by means of NIS Elements BR 3.22 imaging software (Nikon Instruments). Stained cells were counted in at least ten fields per sample (field 0.07 mm<sup>2</sup>, magnification 200×) and quantified as percentage of total counted cells. The intensity of PON2 positivity was semiquantitatively scored as negative (0), moderate (1), good (2) and strong (3). Pictures illustrating negative, moderate and good/strong PON2 intensity were shown in Supplementary Figure 2. The staining score was obtained by multiplying the staining intensity with the percentage of positive cells.<sup>22</sup> Each specimen was analyzed three times.

#### Statistical analysis

Results were analyzed using GraphPad Prism software (GraphPad Softaware Inc., San Diego, California, USA). Differences between groups and correlations with clinicopathological parameters were determined by means of Wilcoxon signed-rank and Mann-Whitney U tests. A p-value <0.05 was considered statistically significant.

#### RESULTS

#### Paraoxonase-2 expression in cutaneous basal cell carcinoma

PON2 immunoexpression was evaluated in both BCCs (Figure 1ab) and controls (Figure 1cd), whose clinicopathological findings are reported in Table 1. No significant association was found between protein level and age (p=0.5388), gender (p=0.4251) and lesion size (p=0.3022). Enzyme immunopositivity was significantly higher in the cytoplasm of BCC cells than in that of controls (staining score: control =  $7.36 \pm 2.46$ ; BCC =  $18.33 \pm 7.41$ ; p<0.0001) (Figure 1e). PON2-related signal was also evident at nuclear envelope level. Interestingly, in nodular BCCs, the staining score was consistent with that of controls (staining score: control =  $8.24 \pm 3.33$ ; nodular BCC =  $8.24 \pm$ 3.42; p $\geq$ 0.05) (Figure 1f), while the infiltrative BCC showed a significant increase of enzyme expression with respect to control (staining score: control =  $6.57 \pm 4.73$ ; infiltrative BCC =  $27.37 \pm 8.98$ ; p<0.0001) (Figure 1g). Moreover, a significantly enhanced protein positivity was evidenced in infiltrative compared to nodular BCCs (staining score: nodular BCC =  $8.24 \pm 3.42$ ; infiltrative BCC =  $27.37 \pm 8.98$ ; p<0.0001) (Figure 1h), thus supporting the hypothesis of a positive correlation between PON2 levels and tumour aggressiveness.

## Paraoxonase-2 expression in cutaneous melanoma

PON2 expression was also assessed both in melanomas and controls by means of immunohistochemistry (Figure 2ab), and related clinicopathological features are shown in Table 1. Our results showed significantly increased protein levels in the cytoplasm of melanoma cells compared to that of controls (staining score: control =  $2.50 \pm 1.32$ ; melanoma =  $58.75 \pm 29.76$ ; p<0.0001) (Figure 2c). PON2 immunopositivity was also found in the nuclear envelope. Furthermore, a statistically significant positive correlation was observed between enzyme expression and clinicopathological findings, such as Breslow thickness (p<0.0001), Clark level (p<0.0001), the presence (p<0.0001) and number (p=0.0082) of mitoses, primary tumor (pT) parameter (p=0.0061) and pathological stage (p=0.0061). In addition, our results showed an

increased PON2 immunoexpression in samples with lymph node metastass (p=0.049), as well as in those without regression (p=0.047) (Figures 3 and 4). On the contrary, the association between enzyme levels and age (p=0.4889), gender (p=0.8161), and the occurrence of both ulceration (p=0.9647) and flogosis (p=0.4831) was not statistically significant. Taken together, these data lead us to suggest a positive correlation between PON2 expression and unfavourable prognosis of melanoma patients, highlighting an interesting prognostic potential for the enzyme.

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#### DISCUSSION

In this work, immunohistochemical analyses were performed to evaluate PON2 expression in tumor and control samples from BCC patients. Moreover, enzyme level was investigated in specimens obtained from patients affected with melanoma, using nevi as controls. Results showed that the enzyme was overexpressed in BCCs compared with controls. Based on different aggressiveness, we found no significant differences in PON2 levels between nodular BCCs and controls. On the contrary, enzyme expression was significantly increased in infiltrative BCCs compared with controls. Interestingly, PON2 was also found to be strongly upregulated in melanoma samples with respect to controls.

Subsequently, the association between PON2 intratumour levels and clinicopathological parameters of BCC and melanoma were explored. There was no significant correlation between enzyme expression and age, gender and diameters related with tumor samples obtained from BCC patients. Conversely, PON2 levels were positively correlated with important melanoma prognostic factors, such as Breslow thickness, Clark level, regression, mitoses, lymph node metastases, pT and pathological stage.

In the work of Wang et al., PON2 immunoistochemical expression was explored in a large number of tumor samples from patients affected with gastric cancer (GC) and control tissue specimens. Furthermore, the correlation between enzyme levels and clinicopathological characteristics of GC patients were evaluated. Results clearly demonstrated PON2 overexpression in GC with respect to normal gastric tissue. Enzyme levels were positively associated with clinical stage, pT, lymph node and distant metastases.<sup>19</sup>

Immunohistochemistry was also used to explore PON2 expression in ovarian cancer tissue specimens, as well as in normal tissue samples. Protein levels were significantly higher in stage I and stage II tumors compared with normal counterparts, while no significant PON2 overexpression was found in stage III and stage IV lesions.<sup>18</sup>

We recently determined enzyme levels in paired tumor and normal bladder tissue specimens, as well as in urinary exfoliated cells from patients affected with bladder cancer (BC) and healthy subjects. PON2 expression was significantly higher in BC compared to normal-looking tissue. Moreover, an inverse correlation was found between urinary enzyme levels and tumor stage of patients affected with BC, thus suggesting PON2 as promising prognostic factor for this neoplasm.<sup>16</sup>

PON2 overexpression led to a decrease of apoptosis-related death in Bcr-Abl-positive K562 chronic myeloid leukemia cells treated with Bcr-Abl tyrosine-kinase inhibitor imatinib. On the contrary, enzyme knockdown significantly enhanced apoptosis rates of imatinib-treated cells. These results suggest that PON2 could participate to the cellular events promoting primary resistance of chronic myeloid leukemia to treatment with targeted drugs. Moreover, PON2 upregulation decreased both caspase-3 activation and ATP reduction in endothelial EA.hy 296 cells treated with chemotherapeutic drug doxorubicin. Similarly, apoptosis induced by treatment with antineoplastic compounds staurosporin and actinomycin D was significantly reduced in EA.hy 296 cells overexpressing PON2.<sup>23</sup>

In our recent study, we explored the effect induced by PON2 gene silencing and overexpression on phenotype of T24 bladder cancer cells. Results demonstrated that the enzyme seems to promote cell viability and migration. Further analyses aimed to evaluate the impact of PON2 knockdown and upregulation on chemosensitivity, in terms of proliferative capacity, ROS production, as well as activation of caspase-3 and caspase-8. Data reported revealed that, under treatment with cisplatin and gemcitabine, enzyme downregulation led to a decrease of T24 cell viability, while it was associated with an enhancement of ROS release and caspase activation. On the other hand, PON2 overexpressing T24 cells treated with chemotherapeutic compounds displayed an increase of cell proliferation as well as a reduction of both ROS production and activation of caspase-3 and -8. Taken together, these results strongly suggest that the enzyme significantly affects proliferative ability and sensitivity to anti-neoplastic drugs of bladder cancer cell.<sup>24</sup> Similarly, PON2 silencing

resulted in significant inhibition of proliferation as well as migration and invasive ability of MKN45 and SGC-7901 GC cell lines.<sup>19</sup>

PON2 was also found to be involved in mechanisms related with radiation resistance in oral squamous cell carcinoma (OSCC) cells. Enzyme displayed a variable expression in different OSCC cell lines. Interestingly, irradiation led to the induction of enzyme expression and cellular response to the treatment was found inversely related to basal PON2 levels. Cells treated with radiation underwent caspase 3/7 activation, which was negatively correlated with endogenous enzyme expression. Conversely, PON2 knockdown was able to enhance radiation-induced apoptosis in OSCC cell lines. All these findings support the hypothesis that PON2 could contribute to protect OSCC cell against irradiation-induced apoptosis.<sup>15</sup>

Enzyme was also able to positively affect glucose metabolism in pancreatic ductal adenocarcinoma (PDAC), in which the enzyme was found to be overexpressed. Analyses performed in PDAC cell line AsPC-1 revealed that PON2 is transcriptionally repressed by tumor suppressor p53. The lack of functional p53, featuring most of the PDACs, is mainly responsible for enzyme overexpression associated with pancreatic cancer, thus allowing PON2 to interact with glucose transporter GLUT1 and facilitate glucose transport within PDAC cell. This condition leads to the reprogramming and optimization of glucose metabolism in pancreatic cancer cell, in order to satisfy energy fueling demand required for a rapid and efficient cell proliferation. Conversely, cellular starvation induced upon PON2 knockdown significantly reduced PDAC cells growth and metastasis.<sup>17</sup> Regarding molecular biomarkers for NMSCs, telomere length (TL) and microRNAs (miRNAs) have been proposed, even though they display weak diagnostic and/or prognostic potential. In BCCs, TL was found to be widely variable compared with that detected in normal skin samples. Moreover, patients affected with BCC exhibited lower miR-34a levels compared to healthy subjects, but an opposite trend was observed in large non-invasive lesions, in absence of lymph node infiltration.<sup>25</sup> Among markers for histological diagnosis of melanoma, HMB-45, Melan-A, Tyrosinase, MITF and S100 proteins are mainly used. However, most of them showed low

sensitivity for advanced stage disease detection and none is able to distinguish between malignat and non malignant melanocytic lesions.<sup>26</sup> Such evidences clearly demonstrated the limit of these biomarkers concerning their use for diagnostic and prognostic purposes.

Our study is the first to to demonstrate PON2 upregulation in both BCC and melanoma, as well as to identify a significant positive correlation between enzyme expression and aggressiveness of these tumors.

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#### LEGENDS

**Figure 1.** PON2 immunoexpression in BCCs. Immunohistochemical staining of PON2 in nodular (a) and infiltrative (b) BCC sections and in their controls (c and d, respectively). Arrow heads indicate PON2 immunopositivity at nuclear envelope level. Values reported in bar diagrams represent the mean staining score  $\pm$  standard deviation. (e) PON2 expression in tumor tissue with respect to controls, considering all BCC cases. (f) Protein levels in nodular BCC. (g) Enzyme immunopositivity in infiltrative BCC. (h) Comparison between PON2 expression in nodular and infiltrative BCC (\*\*\*p<0.0001; n.s. = not significant).

Figure 2. Immunopositivity (a and b) and staining score (c) of PON2 in control nevi and melanomas. Inserts show higher magnification images. Arrow heads indicate PON2 immunopositivity at nuclear envelope level. Reported values represent the mean staining score  $\pm$  standard deviation (\*\*\*p<0.0001).

**Figure 3.** Correlation between PON2 expression in melanoma specimens and clinicopathological findings: Breslow thickness (a), Clark level (b), presence of mitoses (c), number of mitoses (d), lymph node metastasis (e) regression (f), pT (g) and pathological stage (h). Reported values represent the mean staining score  $\pm$  standard deviation (\*p<0.05; \*\*p<0.01; \*\*\*p<0.0001).

Figure 4. PON2 immunohistochemical staining in melanomas showing different clinicopathological features. Enhanced enzyme expression in lesions with high Breslow thickness and Clark level (a) compared to thin and low Clark level melanomas (b). Increased immunopositivity in high-mitotic-rate malignancies (c) with respect to tumors with no mitoses (d). Arrows indicate cells undergoing mitosis. Arrow heads indicate PON2 immunopositivity at nuclear envelope level.

# **LEGENDS TO SUPPLEMENTARY FIGURES**

**Supplementary Figure 1.** Representative images of negative (a, nevus) and positive (b, kidney) controls.

Supplementary Figure 2. Examples of PON2 staining intensity: negative (a, nodular BCC),

moderate (b, melanoma) and good/strong (c, melanoma) intensity. Arrows indicate strong staining intensity.

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# **AUTHORS' CONTRIBUTIONS**

TB contributed to conceiving the study. ES contributed to immunohistochemical evaluation and cowrote the manuscript. VP contributed to immunohistochemical evaluation and co-wrote the manuscript. RC performed statistical analyses. EM selected BBC cases to be included in the study. VB selected melanoma cases to be included in the study. LT contributed to case selection and immunohistochemical analyses. GL contributed to elaboration of data obtained from immunhistochemistry. DS oversaw the results and co-wrote the manuscript. AC participated to cases selection to be included in the study and revised the manuscript. MMB contributed to elaboration of data obtained from immunhistochemistry. CR performed immunohistochemical analyses. GF contributed to conceiving the study. AO contributed to conceiving the study. ME conceived the study and coordinated the research. 

Controls (nevi)

Categories	BCCs	Melanomas
Cases	36	29
Gender		
Males	23	18
Females	13	11
Age		
Mean	68	61
Range	41-83	28-96
Diameter (cm)		
Mean	0.7	n.a.
Range	0.2-1.3	n.a.
Subtype		
Nodular	17	n.a.
Infiltrative	19	n.a.
Breslow thickness (mm)		
Mean	n.a.	1.6
Range	n.a.	0.1-8
Clark level		
Ι	n.a.	1
II	n.a.	5
III	n.a.	10
IV	n.a.	11
V	n.a.	2
Mitotic rate (mitoses/mm <sup>2</sup> )		
No mitoses	n.a.	16
1-4	n.a.	5
≥5	n.a.	8
Regression		
No	n.a.	23
Yes	n.a.	6
Ulceration		
No	n.a.	26
Yes	n.a.	3
Flogosis		
No	n.a.	14
Yes	n.a.	15

Gender			
Males	23	18	15
Females	13	11	12
Age			
Mean	68	61	64
Range	41-83	28-96	37-85
Diameter (cm)			
Mean	0.7	n.a.	n.a.
Range	0.2-1.3	n.a.	n.a.
Subtype			
Nodular	17	n.a.	n.a.
Infiltrative	19	n.a.	n.a.
Breslow thickness (mm)	-		
Mean	n.a.	1.6	n.a.
Range	na	0.1-8	n a
Clark level	11.0.	0.1 0	11.00.
I	na	1	na
Î	n a	5	n a
	n a	10	n a
IV	n a	11	n.a.
V	n a	2	n a
$\frac{v}{Mitotic rate (mitoses/mm^2)}$	11.a.	2	11. <i>a</i> .
No mitoses	no	16	no
	11.a.	5	11.a.
1- <del>4</del> >5	11.a.	0	11.a.
2.) Decreasion	II.a.	o	II.a.
No	<b>n</b> 0	22	<b>n</b> 0
NO Vac	n.a.	23	n.a.
Y es	n.a.	0	n.a.
Viceration		26	
No	n.a.	26	n.a.
Yes	n.a.	3	n.a.
Flogosis			
No	n.a.	14	n.a.
Yes	n.a.	15	n.a.
pl		1-	
1	n.a.	17	n.a.
2	n.a.	3	n.a.
3	n.a.	6	n.a.
4	n.a.	3	n.a.
Lymph node metastases			
NO	n.a.	23	n.a.
N+	n.a.	6	n.a.
Distant metastasis			
M0	n.a.	29	n.a.
M+	n.a.	0	n.a.
Pathological stage (TNM)			
Ι	n.a.	17	n.a.
II	n.a.	6	n.a.
III	n.a.	6	n.a.
Subtype			
Compound	n.a.	n.a.	8
Dormal	na	na	10



Figure 1abcd



Figure 1efgh



Figure 2ab



Figure 2c



\$

1<sup>11</sup>



Figure 3



Figure 4



Supplementary Figure 1 163x71mm (300 x 300 DPI)



Supplementary Figure 2